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Pan-azole resistance in clinical *Aspergillus fumigatus* isolates carrying TR34/L98H from birds and mammals in Belgium

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A B S T R A C T

Aspergillosis causes significant health risks to both birds and mammals. The outcome of these infections is often poor due to delayed diagnosis and treatment failure. We investigated 152 cases of aspergillosis from birds and mammals in Belgium. Most samples originated from the taxonomic orders Artiodactyla (40.1 %) and Columbiformes (19.7 %). Five isolates (3.3 %) showed phenotypical resistance against at least one medical azole. Three of these isolates were pan-azole resistant bearing the TR34/L98H mutation. The predominance of this resistance mutation supports an environmental route for exposure and resistance selection, highlighting the importance of the One Health concept.

1. Introduction

Aspergillosis, primarily caused by the fungus *Aspergillus fumigatus*, poses a significant health risk to both birds and mammals, leading to illness and death. Aspergillosis in birds is quite common and affects birds of all ages and environments. In comparison, aspergillosis is less prevalent in mammals, regardless of the increasing number of immunocompromised animals [1]. Exceptions include canine sinonasal aspergillosis, equine guttural pouch mycosis and bovine mycotic abortion caused by *Aspergillus* spp. [1–3]. Predisposing factors for the development of aspergillosis in birds and mammals consists of immunosuppression, previous debilitating illnesses, stress factors and environmental factors [1,4,5]. These latter factors can consist of contaminated feed, soil and bedding or poor husbandry such as poor ventilation, high humidity, and warm temperatures [1]. The mode of entry is presumed to be oropharyngeal during inhalation, as such the most frequently affected areas are the head region for mammals, and lungs and air sacs in birds [1]. Diagnosis often occurs late and diagnostic tools are scarce, inaccurate or expensive, which limits the chance of timely treatment [6]. Additionally, because of the concurrent severe underlying diseases in mammals, treatment or prophylaxis is challenging [5]. The emergence of azole resistance in *A. fumigatus* in the clinics and the environment has become a major concern. This emergence of resistance also creates additional difficulties for the treatment of aspergillosis in veterinary medicine [7–10]. The pathogen *A. fumigatus* is a perfect example of the interconnectedness of human, animal, and environmental health that are taken into account in the framework of the OneHealth approach [11]. In this study we aimed to contribute to the understanding of the occurrence of azole resistance in veterinary aspergillosis caused by in *A. fumigatus* in Belgium in the OneHealth perspective.

2. Methods

Between 01/2020 and 01/2024, 152 cases of animal aspergillosis caused by *A. fumigatus* were identified by Zoolyx ($n = 70$), Animal health care Flanders ($n = 60$), Ghent University ($n = 15$), the Regional Association for Animal Health and Identification Wallonia (ARSIA) ($n = 4$) and the Scientific Department of Avian Virology and Immunology of Sciensano ($n = 3$). Diagnosis of aspergillosis was based on macroscopic lesions suspected of aspergillosis, detection of fungal hyphae during histological examination, and isolation and identification via culture of the fungus of the affected organs. Necropsy was performed on 131 animals (86.2 %). Cases were identified based on fungal morphology after

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three to five days of growth on malt chloramphenicol (0.5 %; MC) at 37 °C and confirmed using MALDI-TOF MS identification [12].

Data on antibiotic and antifungal treatment were available for 70 animals (46.05 %), however no details on duration or which treatment was used was available. Out of these, 44 (62.86 %) received antibiotic treatment, whereas only 2 (2.86 %) were treated with antifungals. No data were available for 82 animals (53.9 %).

Azole susceptibility testing was performed on all isolates ($n = 152$) using the broth microdilution method following EUCAST guidelines (E. Def. 9.4) to determine the minimum inhibitory concentrations (MICs) to the medical azoles voriconazole, itraconazole, isavuconazole and posaconazole. In the absence of veterinary breakpoints for predicting the clinical response to therapy in mammals and birds, human clinical breakpoints were used (EUCAST v10.0). Sanger sequencing of the *cyp51A* gene, the target gene of the azoles, was performed on the isolates showing phenotypical azole resistance.

3. Results

All isolates were confirmed as *A. fumigatus* by MALDI-TOF MS. *A. fumigatus* isolates were cultured from various animal species and infection sites. Among the avian and mammalian species, the taxonomic orders Artiodactyla ($n = 61$, 40.1 %) and Columbiformes ($n = 30$; 19.7 %) were respectively the most frequently observed, followed by Perissodactyla ($n = 13$, 8.6 %), Charadriiformes ($n = 12$, 7.9 %), Psittaciformes ($n = 7$, 4.6 %), Carnivora (n = 7, 4.6 %) and Passeriformes ($n = 6$, 3.9 %) (Table 1). Most individuals were production animals ($n = 62$, 40.8 %), all belonging to Artiodactyla, while 60 other animals were pets (39.5 %), including 29 pigeons, 13 horses, 6 dogs, 3 grey parrots, 2 macaws, 1 Rosella parrot and one cat. The cases also included 18 wild birds (11.8 %) consisting of 10 common guillemots (*Uria aalge*), one razorbill (*Alca torda*), one Eurasian collared dove (*Streptopelia decaocto*), one Red-throated loon (*Gavia stellata*), one great crested grebe (*Podiceps cristatus*), one northern gannet (*Morus bassanus*) and two other *Passeriformes.* Twelve birds (7.9 %) were sent from zoos and included four Humboldt penguins (*Spheniscus humboldti*), common scoter (*Melanitta nigra),* one freckled duck *(Stictonetta naevosa)* and a tree duck (subfamily Dendrocygninae), one swift parrot (*Lathamus discolor*), one squacco heron (*Ardeola ralloides*), one Bird-of-paradise (family *Paradisaeidae*), one cinereous vulture (*Aegypius monachus*) and one crested oropendola (*Psarocolius decumanus*)(Table 1).

In total, 88 respiratory samples (57.9 %) and 50 samples from the digestive system (32.9 %) were included. Out of the 88 respiratory samples, 64 (72.7 %) originated from avian species, whereas 48/50 samples (96.0 %) from the digestive tract were obtained from cattle. Additionally, six samples were obtained from the reproductive system (four from the uterus and two from the placenta) and eight originated from various other sources: abscess, ear, skin, kidney, pericardium swab, ear canal, tongue swab and a foot abscess (Table 1).

MIC testing identified five isolates (3.3 %) that showed phenotypical resistance against at least one medical azole. No elevated MIC values were present in the other isolates ($n = 147$) for all four medical azoles, showing no evidence of acquired resistance in avian or mammalian populations (Fig. 1). Azole resistance was *cyp51A* mediated in 80 % of resistant isolates.

Among the avian isolates, two azole resistant *A. fumigatus* (ARAf) isolates (2/71, 2.8 %) were isolated from birds living in captivity (pigeons). Both birds received antibiotic treatment but no antifungal treatment. No details were provided about the treatment regimen. They displayed the pan-azole resistant phenotype and carried the TR34/L98H mutation in the *cyp51A* gene (Table 2).

The remaining three ARAf isolates (3/81, 3.7 %) were isolated from mammals: 2 cows and one cat. The cat (IHEM 28552) received antibiotic and antifungal treatment and displayed resistance to voriconazole, isavuconazole and posaconazole. This *A. fumigatus* strain was isolated from an infected ear of a cat. One isolate (IHEM 28550) originating from cattle displayed the pan-azole resistant phenotype and carried the TR34/L98H mutation. The other isolate (IHEM 28429) displayed resistance to posaconazole but was susceptible to itraconazole. Regarding voriconazole and isavuconazole, the MIC value was in the area of technical uncertainty (ATU). The isolate displayed the F46Y, M172V, E427K, N248T, D255E polymorphism. These polymorphisms have been detected in both azole resistant as azole susceptible isolates [15].

4. Discussion

This study showed the presence of pan-azole resistant *A. fumigatus* isolates in veterinary cases. Scarce data is available on azole resistance

Table 1
Number and taxonomy of animals with veterinary aspergillosis according to their origin and sample type.

	Taxonomic order	Origin				Sample type				Necropsy
		Pet	Production	Wild	Zoo	Digestive system	Reproduction system	Respiratory system	Other*	
Avian ($n = 71$)	Accipitriformes ($n = 1$)	0	0	0	1	0	0	1	0	1
	Anseriformes ($n = 4$)	1	0	0	3	0	0	3	1	3
	Charadriiformes ($n = 12$)	0	0	12	0	0	0	12	0	12
	Columbiformes (n = 30)	29	0	1	0	0	0	27	3	30
	Galliformes (n = 3)	2	1	0	0	0	0	2	1	3
	Gaviiformes (n = 1)	0	0	1	0	0	0	1	0	1
	Passeriformes (n = 6)	2	0	2	2	2	0	4	0	6
	Pelecaniformes(n = 1)	0	0	0	1	0	0	1	0	1
	Podicipediformes (n = 1)	0	0	1	0	0	0	1	0	1
	Psittaciformes (n = 7)	6	0	0	1	0	0	7	0	7
	Sphenisciformes (n = 4)	0	0	0	4	0	0	4	0	3
	Suliformes (n = 1)	0	0	1	0	0	0	1	0	0
Mammal ($n = 81$)	Artiodactyla (n = 61)	0	61	0	0	48	3	9	1	60
	Carnivora (n = 7)	7	0	0	0	0	0	5	2	1
	Perissodactyla (n = 13)	13	0	0	0	0	3	10	0	2
Total (n = 152)		60 (39.5 %)	62 (40.8 %)	18 (11.8 %)	12 (7.9 %)	50 (32.9 %)	6 (3.9 %)	88 (57.9 %)	8* (5.3 %)	131 (86.2 %)

* other sample types include: abscess, ear, skin, kidney, pericardium swab, ear canal, tongue swab and foot abscess.

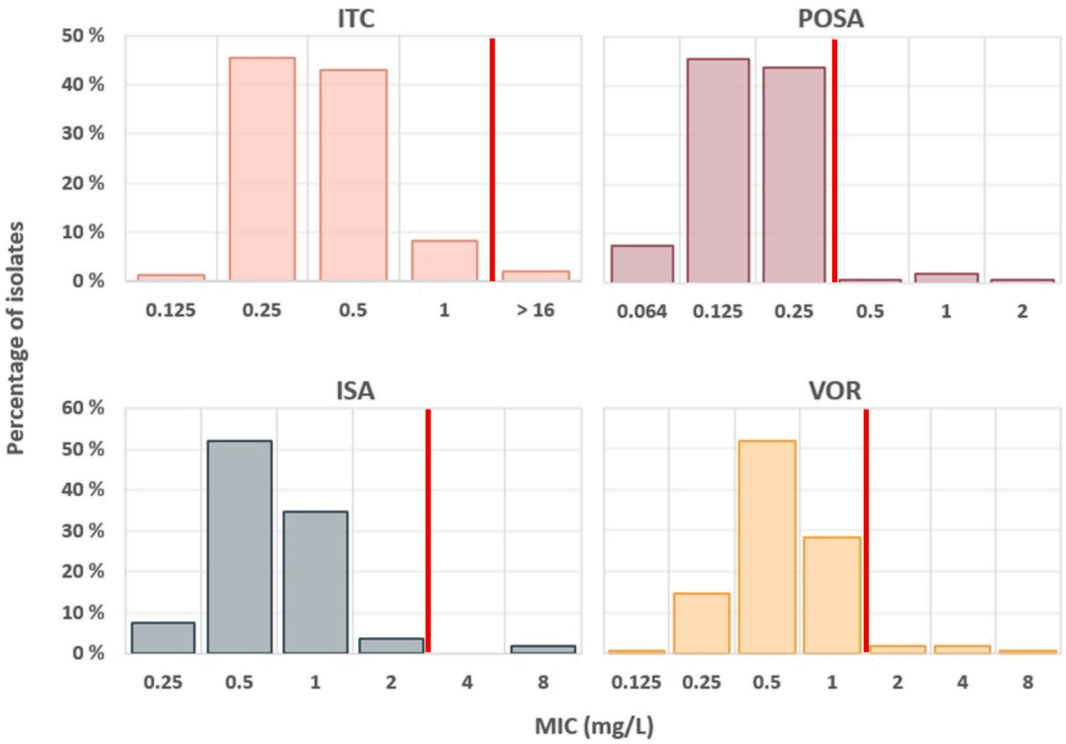

Fig. 1. Azole susceptibility in avian and mammalian *Aspergillus fumigatus* isolates (n = 152). Minimal inhibitory concentration (MIC) values for itraconazole (ITC), posaconazole (POSA), isavuconazole (ISA), and voriconazole (VOR) were determined using broth microdilution following EUCAST protocol E.DEF 9.4 20. No isolates displayed itraconazole MIC values of 2, 4, or 8; therefore, these values are not shown in the graph. The red line represents the clinical breakpoint (EUCAST v10.0). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Minimal inhibitory concentration against medical azoles data and their related *cyp51A* gene sequencing result.

Accession number	Species	Sample type	Year of isolation	MIC (mg/L)[1]				*cyp51A* mutations
				VOR	ITC	ISA	POS	
28,429	Cow	Digestive	**2020**	**2**	0,5	2	**0,5**	F46Y, M172V, E427K, N248T, D255E
28,550	Cow	Digestive	**2020**	**4**	**>16**	**8**	**1**	TR34/L98H
28,552	Cat	Ear	**2021**	**8**	1	2	**2**	no mutation
29,030	Pigeon	Respiratory	**2023**	**4**	**>16**	**8**	**1**	TR34/L98H
29,031	Pigeon	Respiratory	**2023**	**4**	**>16**	**8**	**1**	TR34/L98H

MIC = Minimal inhibitory concentration; VOR = voriconazole; ITC = itraconazole, ISA = isavuconazole; POS = posaconazole. MIC was determined following the EUCAST method for susceptibility testing of moulds (version 9.4). Numbers in bold represents phenotypical resistance according to EUCAST clinical breakpoints for fungi v10.0 [13,14]. The isolates were deposited in the BCCM/IHEM collection under the mentioned accession numbers (https://bccm.belspo.be/about-us/bccm-ihem).

frequency in *A. fumigatus* from animals. However, azole resistance in veterinary medicine might be an increasing concern due to the rise of azole resistance in the environment, since infection generally has an environmental source in veterinary aspergillosis [1]. Here, we observed an overall resistance rate of 3.3 %, with resistant isolates found in birds (2.8 %) and in mammals (3.7 %). The prevalence of resistance in isolates from birds was higher than in other studies on avian aspergillosis [16,17], but lower than described in Humboldt penguins in Belgium [8]. An overall prevalence of ARAf of 11.3 % was observed in veterinary clinical isolates in the Netherlands, which is similar to the frequency observed in humans in the Netherlands [18]. The difference in ARAf prevalence in animals between Belgium and the Netherlands might be explained by the presence of hotspots and the consequently higher environmental prevalence of ARAf in the Netherlands [19], compared to Belgium's 2.6 % [20]. A Belgian surveillance program at the tertiary care center, University Hospitals Leuven, assessed the prevalence of triazole resistance in *A. fumigatus* from complex culture-positive patients in clinical isolates from 2016 to 2020. The surveillance revealed triazole resistance prevalence rates of 8.3 %, 6.7 %, 7.0 %, 7.1 %, and 7.4 % for the years 2016–2020 respectively [21]. In comparison, in the same period, a Dutch national surveillance program reported significant higher triazole resistance prevalence rates of 12.9 %, 14.7 %, 10.5 %, 9.1 % and 8.2 % from 2016 to 2020 respectively (two-sample Wilcoxon rank-sum test; p-value = 0.016 [18].

The observed overall occurrence of resistance was lower than the prevalence reported in human cases in Belgium [21]. Caution is however needed when interpreting the prevalence of resistance data, as these samples were not derived from a systematic monitoring program, potentially introducing bias. Additionally, no clinical breakpoints currently exist for *A. fumigatus* in veterinary medicine. Clinical breakpoints used in human medicine are often applied to interpret susceptibility patterns in animals, however, the predictive value of susceptibility data might be limited due to the difference in anatomical and physiological characteristics [3]. Nevertheless, applying these clinical breakpoints can help indicate the potential presence of *cyp51A* mutations. In this study, 80 % of the resistance mutations was related to the *cyp51A* gene, which is in line with the Belgian human population [21]. One isolate did not display any mutation in the *cyp51A* gene, but other

mechanisms that confer azole resistance in *A. fumigatus* exist and are known to circulate in the environment at low frequencies [22]. The presence of the TR34/L98H mutation in the veterinary isolates, which knows its origin in the environment, suggests a possible link with the environment from which the animals could have inhaled the resistant spores [23]. Here, we did not observe single nucleotide polymorphisms (SNPs) in the *cyp51A* gene, which are generally related to development of resistance through prolonged treatment. Although the current study was restricted by the limited data provided concerning the antibiotic or antifungal treatment regimens, this suggests that, similar to human medicine, the environmental route is dominant in veterinary medicine.

In this study, all isolates were identified as *A. fumigatus* by MALDI-TOF MS. We acknowledge the limitation that species-level identification was not further confirmed by sequencing. However, several studies indicate a very low prevalence of cryptic species, with *A. fumigatus* sensu stricto remaining the predominant causal agent within the *Fumigati* section in birds [24,25]. For instance, Berber et al. and Sabino et al. reported no cryptic species [16,25], while Cateau et al. identified only one cryptic species (*A. nishimurae*) [26].

Generally, treating aspergillosis in animals presents challenges and is associated with poor outcomes [1,2]. The treatment of aspergillosis in animals is often prolonged, and assessing its effectiveness can be challenging. Moreover, in some cases, treatment is no longer viable due to delayed diagnosis. If infection with an azole-resistant *A. fumigatus* strain is suspected, alternative antifungal options such as liposomal amphotericin B or caspofungin may be considered [27]. Considering the increasing rates of azole resistance in the environment and the risk of animal infections through the inhalation of airborne conidia, raising awareness among veterinarians is essential. Prevention through measures such as avoiding the inhalation of conidia by maintaining mold-free husbandry practices or feed is crucial. Do Nascimento et al. found that introducing *A. fumigatus* into waste piles from horse stables can facilitate composting and decrease total coliforms [28]. However, while the transmission of *A. fumigatus* from infected animals to humans is improbable, the potential for a high density of environmental *Aspergillus* conidia in animal housing due to this practice may elevate the risk of inhalation for both horses and humans, posing a significant threat of invasive infections, particularly in hematological and immunocompromised patients [29,30].

Our study demonstrates that azole resistance is present in clinical *A. fumigatus* isolates from birds and mammals in Belgium at a similar frequency as observed in the environment. The predominance of *cyp51A* TR34/L98H resistance mutation supports an environmental route for resistance selection. With the growing concern of azole resistance in both the environment and human medicine, further investigation into azole resistance in veterinary medicine is imperative for implementing effective control measures and maintaining the efficacy of antifungal treatments in veterinary practice.

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CRediT authorship contribution statement

Hanne Debergh: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Roel Haesendonck:** Resources. **Nadine Botteldoorn:** Resources. **An Martel:** Resources. **Frank Pasmans:** Resources. **Claude Saegerman:** Writing – review & editing, Supervision. **Ann Packeu:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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